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Anatomy of a Natural Composite Material

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06	03		Biocomposites, Dopa-proteins, collagen, curing

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

Sessile marine invertebrates deposit specialized protective coatings on extracellular structures such as holdfasts and shells. These coatings, once cured, are reknowned for their stability and durability (1-2 years). The precursors for these coatings are proteins that appear to be present in separate phases in granules that resemble latex particles with interpenetrating polymer networks. We have developed an efficient method based on perchloric acid extraction of one of these precursors - a Dopa-containing protein. This has expedited expansion of our data bank on Dopa-protein sequences to 12 species and 3 phyla. All sequences contain abundant hydroxylated amino acids and all contain Dopa and lysine, which are present in approximately equal concentrations. Glycine and proline seem negatively correlated in the sequences. Specific aims of this proposal are 1) to identify and characterize coating precursor proteins in addition to the Dopa-proteins, 2) to examine interfacial interactions between these various proteins, and 3) to characterize the mechanism of curing.

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ANNUAL REPORT

GRANT #: N00014-84-K-0290 R & T CODE: 441c004

PRINCIPAL INVESTIGATOR: J. Herbert Waite

INSTITUTE: University of Delaware/ College of Marine Studies

GRANT TITLE: Marine Cement: Anatomy of a Natural Composite Material

PERIOD OF PERFORMANCE: 1 Nov 1989 to 31 Oct 1990

OBJECTIVES: A. To characterize the proteins involved in the formation of the mussel byssus, an adhesive structure used by mussels for opportunistic attachment to surfaces, B. To explore the nature of molecular interactions between these proteins at common interfaces,

ACCOMPLISHMENTS (last 12 months): A. The consensus repeat sequences from the polyphenolic proteins of 6 additional organisms have been determined (see Table I), B. Catecholoxidase (MW 38,000) from the byssus of ribbed mussels has been purified, polyclonal antibodies have been prepared, and cell-free translation of its mRNA has been achieved. It is evident from these studies that the precursor of catecholoxidase is a zymogen (MW 70,000) that is activated by a serine-type protease. Prima facie calculations suggest that the enzyme represents up to 20 % (by weight) of the byssal varnish. At this concentration it likely contributes to the structural integrity of the byssus as well as being a catalyst. C. α,β–dehydroDOPA has been identified as a stable intermediate formed from *o*-quinones in the catecholoxidase -catalyzed oxidation of peptidyl-DOPA. D. The effect of polyphenolic protein (from *M. edulis*) adsorbed to stainless steel has been studied under conditions of accelerated corrosion. SS-304L coupons dipped into solutions containing 0.3 mg protein/ mL are protected from pit formation for at least 48 hrs. Controls using other adsorbed proteins provided no protection. Corrosion potential electrochemistry is not adequate by itself to differentiate between corroding and noncorroding surfaces covered with protein films.

SIGNIFICANCE: The mussel byssus consists of several different proteins whose distribution varies depending on location within the structure. The protective varnish coating all parts of the byssus, for example, consists largely of stoichiometric proportions of a DOPA-containing protein(=glue protein) and the enzyme catecholoxidase. The core of the byssus contains mostly collagen (several kinds), and the adhesive pad, mostly polyphenolic protein. The chemical and physical stability of the byssus in sea water makes the interactions between the various proteins of particular interest.

WORK PLAN: (next 12 months): A. Isolate and characterize byssal collagens (MW, pI, antibodies, peptide mapping), B. Make expression library of ribbed mussel *Geukensia demissa* using λgt 11 in rec⁻ *E. coli* strain. Screen library with polyclonal anti-catecholoxidase and enzyme activity. Amplify cDNA positive colony and sequence cDNA. C. Examine molecular interactions between collagen and polyphenolic protein, as well as the polyphenolic protein and catecholoxidase.

INVENTIONS (last 12 months): 1 patent application submitted pertaining to the identification of α,β-dehydroDOPA in polyphenolic analogues treated with catecholoxidase.

Rzepecki, L.M., & Waite, J.H. (1990) Oxidation of DOPA, DOPA derivatives, DOPA-containing polypeptides, and the products thereof. *App. Ser.* #07/493,402.

PUBLICATIONS (last 12 months):

1. Waite, J.H., & Rice-Ficht, A.R. (1989) A histidine-rich protein from the vitellaria of the liver fluke, *Fasciola hepatica*. *Biochemistry* 28, 6104-6110.

2. Waite, J.H., Hansen, D.C., & Little, K.T. (1989) The glue protein of ribbed mussels *Geukensia demissa*: A natural adhesive with some features of collagen. *J. Comp. Physiol.* 159B, 517-525.

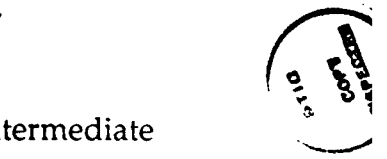
3. Rzepecki, L.M., & Waite, J.H. (1989) A chromogenic assay for catecholoxidase based on the addition of L-proline to quinones. *Anal. Biochem.* 179, 375-381.

4. Waite, J.H. (1990) Phylogeny and chemical diversity of quinone-tanned glues and varnishes (invited review). *Comp. Biochem. Physiol. B*, 97, 19-29.

5. Rzepecki, L.M., Nagafuchi, T., & Waite, J.H. (1990) α,β-DehydroDOPA- A potential intermediate of sclerotization: Oxidative formation. *Arch. Biochem. Physiol.* in press.

6. Rzepecki, L.M., & Waite, J.H. (1990) α,β-DehydroDOPA- A potential intermediate of sclerotization: Kinetics of formation. *Arch. Biochem. Physiol.* in press.

7. Waite, J.H. (1990) Detection of peptidyl-DOPA by amino acid analysis and microsequencing techniques. *Anal. Biochem.* in press.



MOLECULAR DIVERSITY OF DOPA-CONTAINING PROTEINS

The polyphenolic proteins of five marine mussels were isolated using perchloric

acid for the initial extraction from the foot. Perchloric acid was originally introduced for the preferential extraction of lysine- and arginine-rich histones (Johns, 1964), and, presumably, is effective in solubilizing the lysine-rich polyphenolic proteins for similar reasons. The rather surprising feature about this methodology is that yield of DOPA-containing proteins is higher than previous procedures by a factor of 5 to 10, and that it seems to work equally well for polyphenolic proteins of all marine mussel species.

The various proteins resemble those of earlier characterized mussel species in being insoluble in the presence of sodium dodecyl sulfate and in having apparent molecular weights in the range of 80 kD to 130 kD. The amino acid composition of the proteins also reflects properties common to all the known mussel adhesives: high lysine (ranging from 130 res/ 1000 in *M. guyanensis* to 272 res/1000 in *M. modiolus squamosus*), high DOPA (ranging from 89 res/1000 in *M. guyanensis* to 152 res/ 1000 in *M. modiolus squamosus*) and high threonine (ranging from 81 res/1000 in *Brachidontes exustus* to 122 res/1000 in *M. guyanensis*). In contrast to *Mytilus edulis* and *M. californianus*, however, all five mussels of the present study contained elevated levels of glycine (95 res/1000 to 233 res/1000). In this respect, they seem more closely akin to *Geukensia demissa* (Waite et al., 1989) which contained 208 res/1000. All of the mussels have 4-*trans*-hydroxyproline in their polyphenolic proteins; *Septifer bifurcatus* and *M. m. squamosus* have levels that approach or exceed that of *M. edulis*. *M. guyanensis* is unique in maintaining the bulk of its proline in unhydroxylated form (133 res/1000). Another unique aspect about this mussel protein is its high content of histidine (42 res/1000).

Tryptic peptides were generated from each of the four proteins by digestion for 12 to 24 hrs in 0.2 M borate at room temperature. Microsequencing of the peptides bears out the existence of consensus repeats (Table 1). What appears is a potpourri of motifs that harken back to *Mytilus* as well as *G. demissa*. The sequence (T)-G-Y-K, for example, is common to four of the present mussel proteins as well as those of *G. demissa* but not *M. edulis* or *M. californianus* (T-Y-K is as close as these come). *S. bifurcatus* has A-K-P-S and tandem hydroxyprolines which are reminiscent of *Mytilus*, and *M. m. squamosus* has *Mytilus*-like Y*-P*-P* sequences. *B. exustus* sequences contain nothing that is novel, and this is probably their most extraordinary characteristic. Consensus sequences of *B. exustus* appear to be identical with those of *G. demissa*. The two species most certainly belong to distinct genera and inhabit distinct ecological niches. Adhesive protein sequences from two non-molluscan species, *Fasciola hepatica* and *Phragmatopoma californica* are included for diversity's sake. These share the high levels of DOPA, lysine and glycine of other species, but do not contain hydroxyproline.

A plot of the total phenolic (tyrosine + DOPA) amino acid vs lysine contents of each of the proteins gives a straight line with a slope of about one and correlation coefficient of 0.91 (Fig. 1A). We believe this to be suggestive of the putative cross-linking relationship between these two residues. A highly correlated linear relationship also exists between imino acid content vs other amino acids (G + S +

Asx) contributing to beta turns (Fig. 1B). In this case, the slope is negative. This plot suggests that adhesive proteins require beta turns for function, they can achieve these turns by trading imino acid content for other beta formers. To date, only one adhesive *i.e. Mytilus edulis* has been examined for secondary structure. Beta turns are indicated.

Figure 1-A. Relationship between total lysine (lys + hydroxylysine) and phenolic amino acids (DOPA and tyrosine), both in residues per thousand (RPT). Aa (*Aulacomya ater*), As (*Atrina serrata*), Be (*Brachidontes exustus*), Bc (*Bdelloura candida*), Cc (*Choromytilus choros*), Gd (*Geukensia demissa*), Fh (*Fasciola hepatica*), Me (*Mytilus edulis*), Mg (*Mytella guyanensis*), Mms (*Modiolus modiolus squamosus*), Pc (*Phragmatopoma californica*), Sb (*Septifer bifurcatus*), Th (*Trichomya hirsuta*). Proteins in Fig. 1 but not Table 1 have yet to be sequenced.

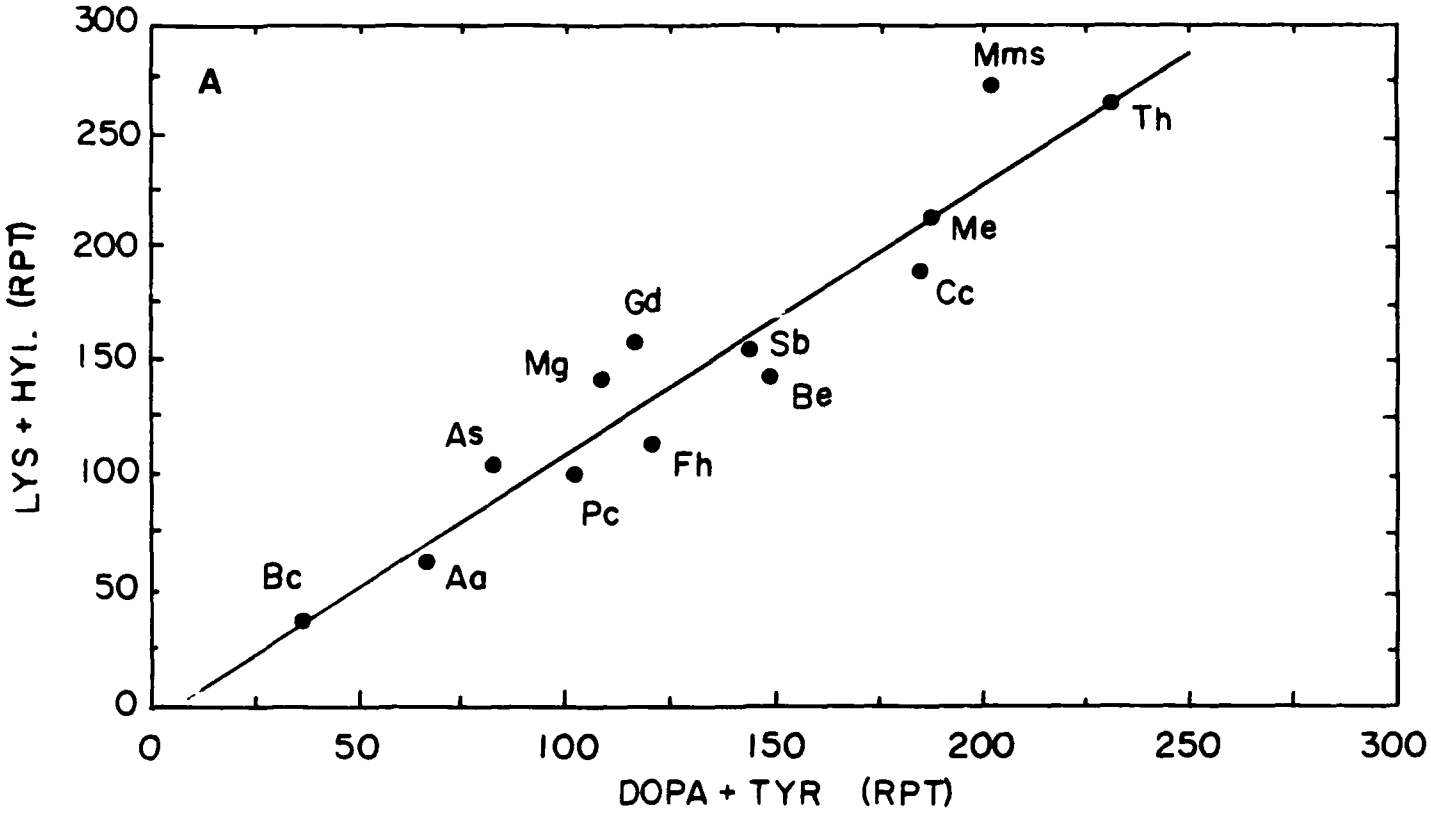
1-B. Relationship between total imino acid content and other beta turn formers such as glycine, serine and aspartic acid/asparagine in residues per thousand.

TABLE 2. Distribution and sequence of some DOPA-containing adhesives and varnishes among the Invertebrata. * indicates occasional hydroxylation, whereas the bold* denotes complete hydroxylation.

SPECIES	Mr	pI	CONSENSUS PEPTIDES (# repeats)
Phylum Mollusca			
Mytilus edulis L. 1758 (Blue mussel)	130 kDa	10	A-K-P-S-Y*-**P***-**P***-T-**Y***-K. (76) A-K-P-T-Y*-K. (15)
Mytilus californianus Conrad, 1837 (Pacific mussel)	85 kDa	10	I-T-Y*-**P***-**P***-T-**Y***-K.-P*-K R-K-P-S-Y*-**P***-**P***-T-**Y***-K. (30)
Septifer bifurcatus (Conrad, 1837) (Bifurcate mussel)	130 kDa	10	Y-**P***-A-K-P-T-S-Y*-G-T-G-**Y***-K. (70)
Geukensia demissa (Dillwyn, 1817) (Ribbed mussel)	130 kDa	8	Q-T-G-**Y***-X-G-Y*-K. (60) X = SA,VP,DP,VL
Modiolus modiolus squamosus Beauperthuy, 1967 (False tulip mussel)	100 kDa	>10	S-Y-Y*-P*-P*-K. (91)
Brachidontes exustus (Linne, 1758) (Scorched mussel)	90 kDa	8	same as *G. demissa*. (60)
Mytella guyanensis (Soot Ryen) (Ecuadorean mussel)	130 kDa	10	A/S-H-K-P-Y*-T-G-**Y***-K. (>70)
Aulacomya ater (Shoe mussel)	100 kDa	?	A-G-Y*-G-G-X-K.* (?) X = V,L
Trichomya hirsuta (Lamarck 1819)	120 kDa	>10	X-Y*-Y*-P*-K. (50) X = S, T, G G-Y*-G-X-K. (25) X = S, A
Phylum Annelida			
Phragmatopoma californica (Fewkes) (Reef building worm)	20 kDa	8.2	V-G-G-Y*-G-Y*-G-A-K. (31) A-L-G-G-**Y***-G-A-G-A-H-P-A-V-H-K. (20)
Phylum Platyhelminthes			
Fasciola hepatica L. (Liver fluke)	31 kDa	7.2	X_1-Y*-X_2-X_3-Y*-X_4-K. (33)
	17 kDa	6.9	G-X, where X = Y*, S or H. (34)

X_1= variable; X_2= usually D, A, E, G; X_3= usually S, D, G; X_4= usually G.

Fig 1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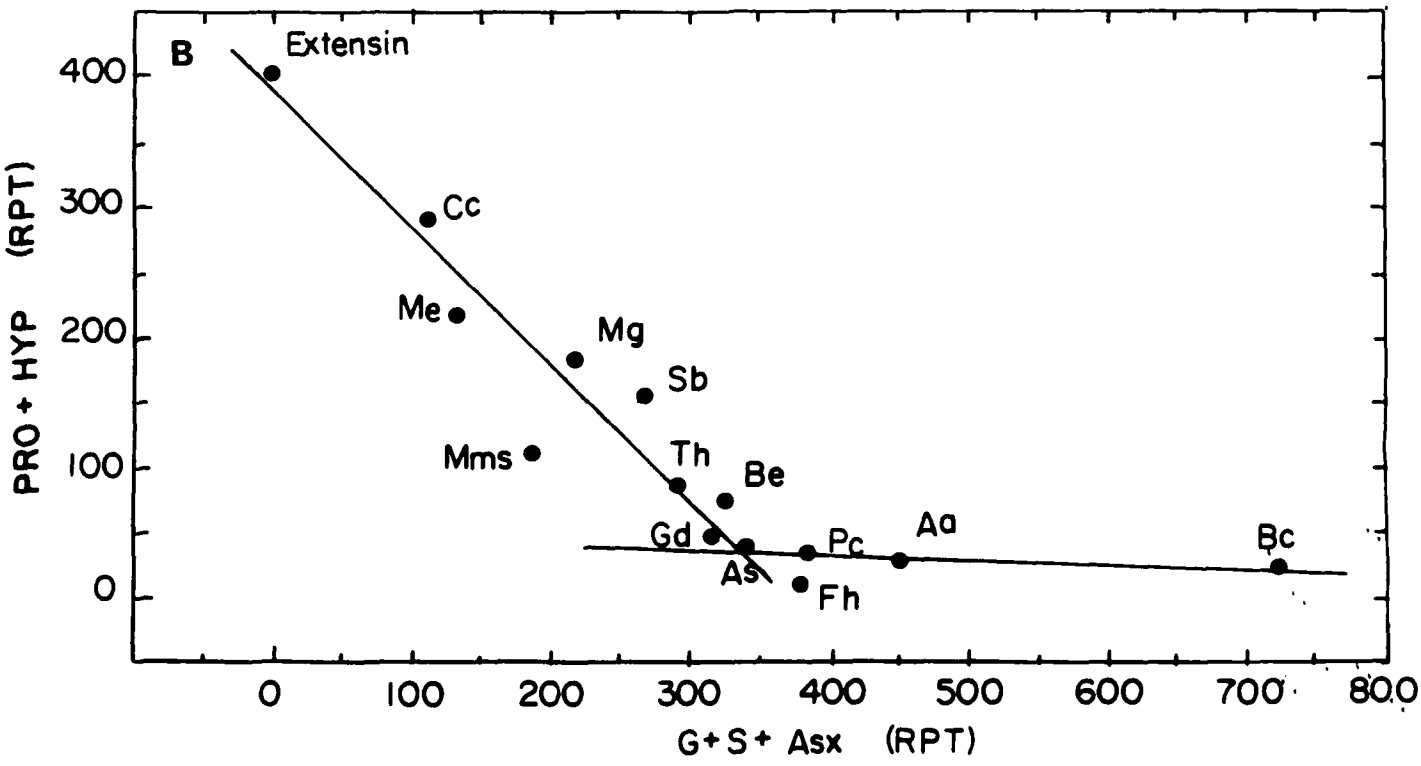
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